IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

DOCKET NUMBER	ANTICIPATED CLASSIFICATION OF THIS APPLICATION:		PRIOR APPLICATION	
	CLASS	SUBCLASS	EXAMINER	ART UNIT
7933.36US01			A. Navarro	1645

CERTIFICATE UNDER 37 CFR 1.10:
"Express Mail" mailing label number: EL435536121US
Date of Deposit: August 9, 1999

I hereby certify that this paper or fee is being deposited with the U.S. Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to Assistant Commissioner for Patents, Washington, D.C. 20231.

By: Hassen Buie
Name: Hassen Buie

CONTINUATION APPLICATION UNDER 37 C.F.R. § 1.53(b)

BOX PATENT APPLICATION
Assistant Commissioner for Patents
Washington, DC 20231

Dear Sir:

This is a request for filing a continuation application under 37 CFR § 1.53(b) of Serial No. 08/691,484, filed on August 2, 1996 entitled NOVEL VACCINES AND PHARMACEUTICAL COMPOSITIONS USING MEMBRANE VESICLES, AND METHODS FOR PREPARING SAME by the following inventor(s):

Full Name Of Inventor	**Family Name** KADURUGAMUWA	**First Given Name** Jagath	**Second Given Name** L.
Residence & Citizenship	**City** Guelph	**State or Foreign Country** Canada	**Country of Citizenship** Canada
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Full Name Of Inventor	**Family Name** BEVERIDGE	**First Given Name** Terry	**Second Given Name** J.
Residence & Citizenship	**City** Elora	**State or Foreign Country** Canada	**Country of Citizenship** Canada
Post Office Address	**Post Office Address** 101 Chalmers Street	**City** Elora	**State & Zip Code/Country** Ontario N0B 1S0 Canada

1. ☒ Enclosed is a copy of the prior application; including the specification, claims, drawings, and any amendments referred to in the oath or declaration filed to complete the prior application. (It is noted that no amendments referred to in the oath or declaration filed to complete the prior application introduced new matter therein.) The continuing application is as follows: 73 pages of specification, 17 claims, 1 pages of abstract, and 35 sheets of drawings. **The inventors' declaration is deferred.**

   ☒ The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

2. ☐ Cancel original claims _____ of this application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

3. ☒ The filing fee is calculated below:

CLAIMS AS FILED

NUMBER FILED	NUMBER EXTRA		RATE	FEE
TOTAL CLAIMS: 17 -20	0	x	$0.00	$0.00
INDEPENDENT CLAIMS 4 -3	1	x	$39.00	$39.00
			BASIC FILING FEE:	$760.00
			TOTAL FILING FEE:	$799.00

☐ A Verified Statement that this filing is by a small entity is already filed in the prior application.

☐ A Verified Statement that this filing is by a small entity is attached.

3. ☒ Payment of fees:
   - ☐ Attached is a check in the amount of $.
   - ☐ Please charge Deposit Account No. 13-2725.
   - ☒ PAYMENT OF THE FILING FEE IS BEING DEFERRED.

5. ☐ The Commissioner is hereby authorized to charge any additional fees as set forth in 37 CFR §§ 1.16 to 1.18 which may be required by this paper or credit any overpayment to Account No. 13-2725.

6. ☒ Amend the specification by inserting before the first line the sentence:

> "This application is a Continuation of application Serial No. 08/691,484, filed August 2, 1996, which claims benefit under Title 35, United States Code § 119(e) of Provisional application No. 60/001,903. Filed 4 August 1995, which application(s) are incorporated herein by reference."

7. ☐ A set of formal drawings (_____ sheets) is enclosed.

8. ☐ Priority of application Serial No. , filed on in , is claimed under 35 U.S.C. 119.

☐ The certified copy has been filed in prior application Serial No. _____, filed _____.

9. ☒ The prior application is assigned of record to University of Guelph located at Guelph, Ontario N1G 2W1 Canada.

10. ☒ The Power of Attorney in the prior application is to:

Merchant & Gould P.C.
3100 Norwest Center
90 South Seventh Street
Minneapolis, MN 55402-4131

11. ☐ A preliminary amendment is enclosed. (Claims added by this amendment have been properly numbered consecutively beginning with the number next following the highest numbered original claim in the prior application.)

☐ Fee for excess claims is attached.

12. ☒ A petition and fee has been filed to extend the term in the prior application until August 9, 1999. A copy of the petition for extension of time in the prior application is attached.

13. ☐ The inventor(s) in this application are less than those named in the prior application and it is requested that the following inventors identified above for the prior application be deleted:

14. ☐ Also Enclosed:

15. ☒ Address all future communications to the **Attention of Douglas P. Mueller** (may only be completed by attorney or agent of record) at the address below.

16. ☒ A return postcard is enclosed.

Respectfully submitted,

KADURUGAMUWA et al

By their Attorneys,

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(612) 332-5300

Dated: August 9, 1999

By

Douglas P. Mueller
Reg No. 30,300
DPM:vvh

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	KADURUGAMUWA et al.	Examiner:	A. Navarro
Serial No.:	08/691,484	Group Art Unit:	1645
Filed:	August 2, 1996	Docket No.:	7933.33US01
Title:	NOVEL VACCINES AND PHARMACEUTICAL COMPOSITIONS USING MEMBRANE VESICLES, AND METHODS FOR PREPARING SAME		

CERTIFICATE UNDER 37 CFR 1.8:
I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail, with sufficient postage, in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231 on August 9, 1999.

Douglas P. Mueller

PETITION FOR EXTENSION OF TIME

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

In accordance with the provisions of 37 C.F.R. §1.136(a), it is respectfully requested that a 3–month extension of time be granted in which to respond to the outstanding Office Action mailed February 9, 1999, said period of response being extended from May 9, 1999 to August 9, 1999.

Our check in the amount of $870.00 is enclosed to cover the required extension fee.

Small Entity is hereby disclaimed.

Respectfully submitted,
KADURUGAMUWA et al.
By their Attorneys,

MERCHANT & GOULD P.C.
3100 Norwest Center
90 South Seventh Street
Minneapolis, Minnesota 55402
(612) 332–5300

Dated: August 9, 1999

By
Douglas P. Mueller
Reg No. 30,300
DPM:vvh

IN TH[redacted]D STATES PATENT AND TRADEM[redacted]ICE

Applicant:	KADURUGAMUWA et al.	Examiner:	A. Navarro
Serial No.:	08/691,484	Group Art Unit:	1645
Filed:	August 2, 1996	Docket:	7933.36US01
Notice of Allow. Date:	NA	Batch No.:	NA
Due Date:	August 9, 1999		

Title: NOVEL VACCINES AND PHARMACEUTICAL COMPOSITIONS USING MEMBRANE VESICLES, AND METHODS FOR PREPARING SAME

CERTIFICATE UNDER 37 CFR 1.8: The undersigned hereby certifies that this Transmittal Letter and the paper, as described herein, are being deposited in the United States Postal Service, as first class mail, with sufficient postage, in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on August 9, 1999.

By: _____
Douglas P. Mueller

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

We are transmitting herewith the attached:

☒ Transmittal Sheet in duplicate containing Certificate of Mailing
☒ Request for Extension of Time for 3 month(s) and fee of $870.00
☒ Return postcard

Please consider this a PETITION FOR EXTENSION OF TIME for a sufficient number of months to enter these papers, if appropriate. Please charge any additional fees or credit overpayment to Deposit Account No. 13-2725. A duplicate of this sheet is enclosed.

MERCHANT & GOULD P.C.
3100 Norwest Center, Minneapolis, MN 55402
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By: _____
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PRODUCTION, CHARACTERIZATION AND CONTROL OF MenB-VACCINE «FOLKEHELSA»: AN OUTER MEMBRANE VESICLE VACCINE AGAINST GROUP B MENINGOCOCCAL DISEASE

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SUMMARY

A vaccine against serogroup B meningococcal disease has been prepared from a B:15:P1.7,16 meningococcal strain (44/76) by fermentor growth and extraction of the bacteria with the detergent deoxycholate. Outer membrane vesicles (OMV) were purified by ultracentrifugation and adsorbed to aluminium hydroxide adjuvant. OMV contained the major class 1, 3, 4 and 5 proteins and some minor high molecular weight protein components. Relative to protein, the vaccine also contained about 8% phospholipid, 7% lipopolysaccharide and 16% deoxycholate. The product was generally non-pyrogenic to humans in ordinary doses and was highly immunogenic in mice and humans. Production and control steps, physical, chemical and immunological data for the vaccine are described.

Key words: Bacteriological techniques; Neisseria meningitidis; Safety; Quality control; Vaccines

INTRODUCTION

During the last 16 years an estimated incidence of 6,000 cases and approximately 500 deaths have been caused by *Neisseria meningitidis* in the Norwegian population of 4.3 million. About 30% of the cases were in the age group 13-20 years, and almost 80% were caused by serogroup B strains (1), mainly belonging to clones of the ET-5 com-

plex, with serotype 15 and serosubtype P1.7,16 (2). Similar epidemics or hyperendemic situations caused by ET-5 group B meningococcal strains, but with other serotypes and subtypes, have been observed in Cuba, Spain, Brazil and Chile (2). Serogroup B meningococci are the most common cause of meningococcal disease (MCd) in interepidemic situations in industrialized countries (3).

In contrast to the A and C capsular polysaccharides from meningococci, the group B polysaccharide (B-PS) is only weakly immunogenic and does not induce bactericidal antibodies in humans (4). A number of possible explanations have been given for the inadequate immunogenicity, including the similarity of B-PS to sialic acid moieties in human tissues (5).

Several studies have revealed correlation between resistance to clinical MCd and bactericidal or opsonizing antibodies to both capsular and subcapsular meningococcal antigens (6,7). These antibodies also persist long after ended MCd (7), and recurrence of MCd in the same persons is very rare, except in cases of complement deficiency.

Meningococcal outer membranes contain a number of antigens which induce bactericidal antibodies in both animals and humans (8,9). Class 1 and class 3 proteins have immunodominant epitopes which elicit highly specific serotype and subtype antibodies. The use of an infant rat passive protection model indicated that antibodies to the class 1 and 3 proteins are promising vaccine candidates (10). Other studies suggested that certain epitopes of the class 4 protein may induce blocking antibodies (11). The class 5 proteins, although highly immunogenic, are normally considered of less utility in a vaccine, due to their variability. However, a protein named 5C by Achtman *et al.* (12) was present in more than 50% of the strains examined (13). This protein elicited bactericidal antibodies in humans (14,46). The class 5 protein P5.5 has also been shown to be present in about 50% of group B meningococcal strains studied in Norway (15).

Meningococcal lipopolysaccharide (LPS) is also a potential vaccine antigen. Monoclonal antibodies (MoAbs) against LPS afforded passive protection in the infant rat model (16). The toxicity of LPS has discouraged the development of an LPS vaccine, and most vaccine producers try to avoid LPS in their vaccines. However, the toxicity was reduced when LPS was tightly bound to outer membrane proteins (OMP) (17). The detergent deoxycholate (DOC) (18) and adsorption to aluminium hydroxide also reduced the toxicity, and thereby made it possible to produce safe vaccines with immunogenic amounts of LPS.

It is not clear if any single one of these possible vaccine antigens is crucial for immune protection. The humoral immune response after natural infection and vaccination was directed towards a number of meningococcal antigens and showed significant variation between individuals (19,20). Thus, any antigen-antibody reaction, arising from one of the vaccine candidates, which results in complement activation, may contribute to ultimate bacterial killing, opsonization and host protection. On this background, we found it reasonable to include nearly the whole antigenic mosaic of the meningococcal outer membrane in the vaccine.

Formulation of the vaccine is also of great importance. Studies have shown that the physical form and solubility of OMP influenced their immunogenicity (21). When membrane proteins were presented in multimeric forms, *e.g.* in liposomes or protein micelles, they had higher immunological activity than in the monomeric form (22). They are also more likely to elicit antibodies to epitopes relevant for protection.

The antibody responses in animals and humans were improved by adsorption of OMP to $Al(OH)_3$ (23). Terlink *et al.* (24) showed that detergents, *e.g.* DOC, improved solubility and immunogenicity of OMP, and that detergent and aluminium adjuvant had synergistic effects.

We developed a complex vaccine and tried to conserve the antigenic mosaic as native as possible in vesicles of intact outer membrane. Strongly denaturating purification steps have consequently been avoided. The phospholipid and LPS present presumably supported the vesicle structure and the formulation was designated outer membrane vesicles (OMV).

We based the vaccine production on one single strain, a clinical disease isolate representative of the Norwegian epidemic. However, as the vaccine contains a number of cross-reacting antigens, a protection trial might indicate the relative importance of type and subtype specific antigens versus cross-reactive antigens for protection (15).

MATERIALS AND METHODS

Bacterial strain

The *Neisseria meningitidis* production strain, 44/76, was a Norwegian isolate from a fatal case of meningitis and septicemia in 1976 (25). A seed-lot system was established at the National Institute of Public Health, Norway, in 1986. The master seed had undergone few laboratory passages since isolation from the patient. The working seed-lot was kept in Greaves' solution (5% bovine serum albumin, 5% Na-glutamate and 10% glycerol) at -70°C. Our vaccine strain was characterized as B:15:P1.7,16:L3,7. The strain also reacted with MoAbs against the class 5 protein 5.5 (15-1-P5.5) and the 5C protein (B306) from Drs. Zollinger and Achtman (12), respectively.

Vaccine production

The production process is based upon the methods of Zollinger *et al.* (26) and Helting *et al.* (27). The bacteria were grown in a 20 l laboratory fermentor (MBR Bio Reactor AG, Switzerland) with stirring and aeration at 36°C until early stationary phase (7 hrs). A modified Frantz' medium (28) was used with 0.1% yeast extract dialysate and 3% casamino acids (Difco, USA). The culture was kept at 4-8°C overnight and harvested by centrifugation (5,000 × g; 15 min; 4°C). The biomass was extracted by magnetic stirring (30 min) at room temperature in 0.1M Tris-HCl, pH 8.6, containing 10 mM EDTA and 0.5% DOC. The ratio of buffer to biomass was 5:1 (v/w). After centrifugation (20,000 × g; 30 min; 4°C) the extraction was repeated with a buffer volume reduced to one third. The combined supernatants were ultracentrifuged (125,000 × g; 2 hrs; 4°C), and the OMV pellet was resuspended in 50mM Tris-HCl buffer, pH 8.6, containing 2 mM EDTA, 1.2% DOC and 20% sucrose. After a second ultracentrifugation step OMV were homogenized in 3% sucrose. Dilution was done in 3% sucrose to a protein concentration of 0.2 mg/ml and the vesicles adsorbed to $Al(OH)_3$-gel (Superfos, Denmark) in a protein/adjuvant ratio of 1:67 (w/w). The final pH was 6.7. All solutions, used in the production process, except the medium, contained 0.01% thiomersalate.

Vaccine characterization

Physical: Before adsorption to adjuvant, OMV were examined by transmission electron microscopy. Droplets of the specimen were applied to glow discharged, carbon filmed copper grids and negatively stained with sodium phosphotungstic acid, pH 7.0.

Chemical: Protein concentration was determined by a modified Lowry method (29). Quantitation of other components was done relative to the protein concentration. The vaccine components were analysed by electrophoresis using polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS-PAGE) (30). Proteins were stained with Coomassie Brilliant Blue (CBB) and LPS with silver stain (17). Estimations of relative amounts of stained components were done by a Shimadzu CS-930 Chromato Scanner, Japan.

For determination of LPS, purified meningococcal LPS of strain 44/76 was used as reference. LPS was measured by gas-chromatographic (GC) determination of the LPS-specific 3-hydroxy fatty acids released by methanolysis and extracted into hexane before trifluoracetylation (31). LPS was also estimated by colorimetry of 2-keto-3-deoxyoctonate (Kdo) in ethanol-precipitated OMV. Mild hydrolysis with 0.5 M HCl at 100°C for 30 min was used to prevent Kdo degradation (32), followed by the thiobarbituric acid method of Osborn (33).

Phospholipids were estimated by GC of fatty acids released by methanolysis (34). DOC was determined by the Enzabile® kit from Nycomed, Norway. DNA was measured by a method based on the Fluorochrome Hoechst 33258 (35). Al and Hg were determined by a Perkin-Elmer 5100 PC atomic absorption spectrophotometer, using an oxidizing acetylene/air flame for Hg and acetylene/nitrous oxide flame for Al. To the samples for Al determination, HNO_3 was added to dissolve hydroxide, and 0.1% K (as KCl) to prevent ionization in the flame.

Immunological: The antigenic properties of the vaccine were evaluated on immunoblots (30) with MoAbs and selected human postvaccination sera. The detergent Empigen BB (0.25%) was present during incubations with some MoAbs to restore antibody binding to SDS denatured epitopes (36).

The amount of B-PS in OMV was analysed using ELISA and a group B specific MoAb (2-1-B) from Dr. Zollinger. Standard curves were made by adding known amounts of B-PS to a fixed amount of OMV.

Controls for batch release

Product control: Quality control (QC) tests used are schematically presented in Table 1. In addition to the tests described above, the QC-tests also included tests for sterility, abnormal toxicity, rabbit pyrogenicity and bacterial endotoxins. All these tests were performed according to the European Pharmacopoeia 2nd ed. Biologically active endotoxin was also measured by a LAL (Limulus Amoebocyte Lysate) chromogenic substrate method (37).

We used antibody response in mice as a quantitative determination of the vaccine's immunizing ability (*i.e.* potency assay). Groups of 18 Bom: NMRI (outbread) mice were given vaccine s.c. in doses of 2 or 4 μg protein. The mice were revaccinated three weeks later and bled by heart puncture two weeks thereafter. Immune responses were evaluated in ELISA using OMV from strain 44/76 as coating antigen.

Test for efficacy of antimicrobial preservation was performed according to the European Pharmacopoeia Commission PA/PH/Exp.1CM/T(89)7.

Characterization and quality control were done on sub-batches, OMV bulk (unadsorbed) and the final product, but control of abnormal toxicity and efficacy of

Table 1
Quality Control of MenB-Vaccine «Folkehelsa»

Schematic presentation; see text for stage and frequency of testing

1. SAFETY
- Endotoxin quantitation: GC, LAL-test, rabbit pyrogenicity
- Abnormal toxicity

2. PURITY
- Protein pattern: SDS-gel, CBB & silver stain
- Test for sterility

3. IDENTITY
- Main proteins and LPS: SDS-gel & immunoblotting
- Vesicle morphology: Electron microscopy

4. POTENCY
- Immunogenicity in mice: ELISA

5. QUANTITATION OF «ADDITIVES»
- Adjuvant: Atomic absorption spectroscopy
- Preservative: Atomic abs. & microbiological efficacy

preservative was done on the final product only. DNA and DOC were not determined in the final adsorbed product. Al and Hg were measured in bulk samples and in the final product. For identity testing of final vials, SDS-PAGE was done after treatment with 0.1% EDTA at 37°C for about 16 hrs.

Environmental control: Quality assurance of aseptic production was provided by an environmental control programme. The «Critical areas» of the premises (*i.e.* rooms where the product was exposed to air), were set to meet class A/B in the EEC-guidelines (38). Controls included measurements with a Hiac/Royco mod. 5250 airborne particle counter and bacterial sampling with a Casella bacterial sampler MK 2. Work operations were also monitored by «settle plates» and «finger dabs». Evaluation of the results and establishment of limits for warning and for exclusion were based on «Environmental Contamination Control Practice» (39) and previous monitoring results from the premises.

Throat cultures of production personnel were taken weekly and in connection with any accidental spillage. Contamination controls, performed at several stages in the production process according to standard operating procedures, included microscopy of Gram stained samples and samples for agar plate cultivation.

RESULTS AND DISCUSSION

Production

Average yield was 15 g biomass (wet weight) per l medium, corresponding to about 225 g biomass per production cycle. After extraction and purification about 2 mg protein/g biomass was obtained. This corresponded to 450 mg protein from one production cycle. The final yield was about 13,000 vaccine doses of 25 μg protein each. One production cycle required 2-3 persons for three days (about 100 hrs of work). Normally one cycle was performed weekly. OMV were stored for a few weeks at 4-8°C before pooling and adsorption. At all stages 0.01% thiomersalate was present.

For a final vaccine batch, OMV from 5-10 production cycles (sub-batches) were pooled. The final (adsorbed) vaccine was stored in vials at 4-8°C until use. Five final vaccine batches were used in the clinical trial with Norwegian teenagers (40).

Figure 1
Electron micrograph of outer membrane vesicles (OMV), *i.e.* unadsorbed MenB-vaccine «Folkehelsa». Magnification 60,000.

Characterization and control

Before adsorption to $Al(OH)_3$ the OMV formed a stable colloidal suspension. The electron micrograph (Fig. 1) shows that the vaccine contained mainly intact vesicles with an average diameter of 100 nm.

The chemical composition is shown in Table 2. In addition to proteins, OMV consisted mainly of phospholipid, LPS and DOC. Measurements of B-PS in ELISA

Table 2
Composition of MenB-Vaccine «Folkehelsa»

Substance	Concentration	Relative to protein µg/100 µg (range)
Protein	50 µg/ml	
LPS	3.5 µg/ml	7 (5-9)
Phospholipid	4 µg/ml	8 (5-12)
DNA	0.2 µg/ml	0.4 (0.16-0.5)
DOC	8 µg/ml	16 (11-22)
B-PS	<0.13 µg/ml	<0.25
$Al(OH)_3$	3.3 mg/ml	
Sucrose	18 mg/ml	
Thiomersalate	0.1 mg/ml	

- One human dose: 0.5 ml (25 µg protein).

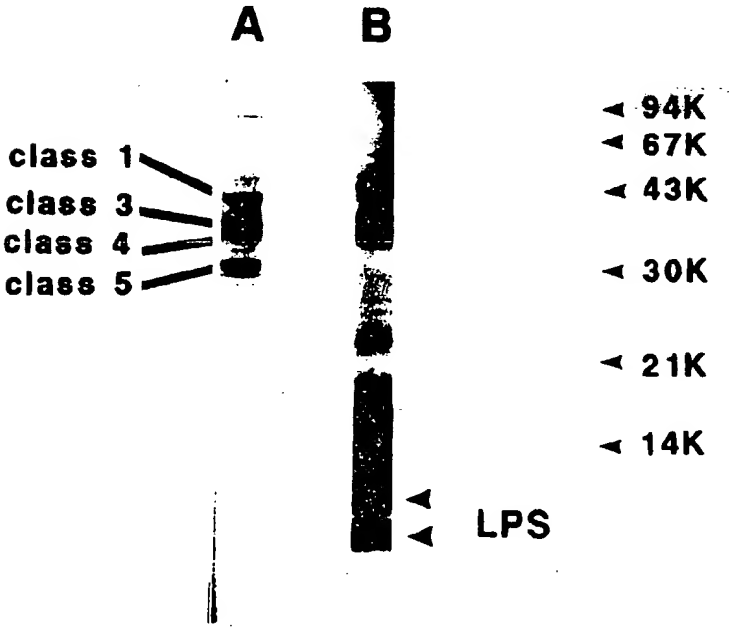

Figure 2
OMV separated in SDS-gel, stained with CBB (A) or silver stain (B). About 2 µg protein per lane. The positions of molecular weight markers are shown to the right and protein class designations to the left.

Table 3
Protein Pattern*

Protein	kDa	Amount (% by scanning)	Range
«High mol. wt.»	80	2	1-3
Class 1	42	33	27-35
Class 3	37	38	34-44
Class 4	34	12	10-17
Class 5	31	15	9-19

* SDS-PAGE analysis of sub-batches. Scanning of CBB-stained gels

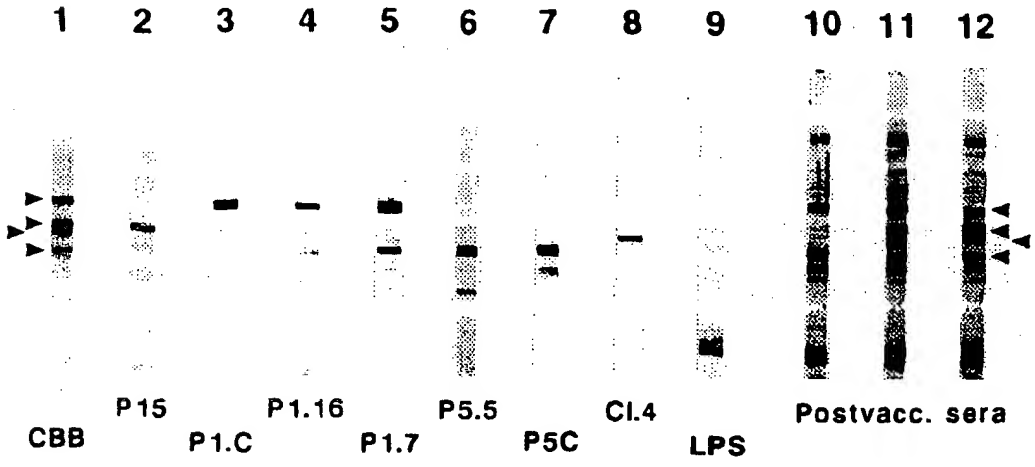

Figure 3
Immunoblots of OMV (about 2 μg protein per lane) with MoAbs (lanes 2-9) and post-vaccination sera (lanes 10-12), developed with peroxidase-conjugated antibodies. OMV were boiled in sample buffer prior to electrophoresis. Lane 1 shows the major proteins stained with CBB. The arrows indicate from top to bottom the positions of the class 1, 3, 4 and 5 proteins, respectively. MoAbs were against serotype 15 protein (lane 2), a common class 1 protein determinant (lane 3), subtypes P1.7 and P1.16 (lanes 4 and 5), class 5.5 and 5C proteins (lanes 6 and 7), class 4 protein (lane 8) and LPS (L3,7,9) (lane 9). IgG antibody binding patterns of three different postvaccination sera are shown in lanes 10-12. The MoAbs were diluted 1:1,000 to 1:20,000; the human sera 1:200. Binding of MoAbs in lanes 2 and 3 was detergent dependent.

demonstrated that the level of B-PS was below the detection limit, *i.e.* less than 0.25% relative to protein.

The pattern of a typical OMV batch after CBB or silver staining of SDS-gels can be seen in Fig. 2. The class 1, 3, 4 and 5 proteins dominated in the CBB stained gel (Fig. 2A). The relative amounts of the different proteins showed small variations among the batches (Table 3). The class 1 and 3 porin proteins each constituted about 30-40% of the total proteins and the class 4 and 5 proteins about 15% each. Other uncharacterized components could be seen, *e.g.* some band(s) with molecular weight of about 80 kD, estimated to 2% of the total proteins. Silver staining demonstrated two dominant bands in the LPS range (Fig. 2B).

The reaction of an OMV preparation with different antibodies is shown in Figure 3. MoAbs against class 1, 3, 4, and 5 proteins were bound. The weaker immunoreactive bands, below the class 1 protein band (lanes 3-5), were absent when boiling of OMV in sample buffer was omitted before electrophoresis. The additional bands were probably caused by cleavage of aspartyl-prolyl bonds during the boiling treatment (41). A similar fragment was also observed by Zollinger *et al.* (42).

Three postvaccination sera, collected six weeks after the second dose, showed individual binding patterns (Figure 3; lanes 10-12). They also reacted with several other antigens and may therefore be used to look for possible differences between OMV batches. The strong response to minor amounts of high molecular weight proteins should be noted. Immunoblotting studies showed no distinct differences between batches or sub-batches.

The antibody response in mice revealed stronger immune response for 4 than 2 μg protein. No significant difference was detected between the five batches used in the clinical trial. The immunogenicity did not decline significantly after two years of storage at 4-8°C, nor was any change in toxicity detected.

GC analyses demonstrated about the same contents of LPS and phospholipid, (Table 2). LPS determination by GC of 3-hydroxy fatty acid and by colorimetry of Kdo gave almost identical results (5-9%). LAL reactivity of OMV gave on the average 350 EU/μg protein, using *E. coli* O55:B5 LPS (9 EU/ng; Whittaker Bioproducts, USA) as reference. This corresponds to an activity of 5 EU/ng for OMV LPS. In comparison purified meningococcal LPS gave about 25 EU/ng LPS. The difference between LAL reactivities in the two types of products was expected, but is smaller than the 20-40-fold difference reported by Tsai *et al.* (17).

When the pyrogenic responses of OMV and purified LPS from strain 44/76 were compared in rabbits, a distinct difference was observed. The pyrogenic effect of LPS in OMV was only one hundreth of the effect of the same amount of purified LPS. Thus, 70 ng LPS in OMV (per kg rabbit) and 0.5 ng purified LPS both gave an average total temperature rise in three rabbits of 1.6-1.7°C. Adsorption of the vaccine reduced the pyrogenicity still further to about 1.3°C for the final vaccine. Low pyrogenicity of LPS in OMV vaccine has been reported by others (17,21). Tsai *et al.* (17) found rabbit pyrogenicity reduced by a factor of ten for LPS in OMV-like preparations. The significantly lower pyrogenicity in our vaccine may in part be due to the rather high content of DOC and phospholipid (see below).

The production method used did not include a specific step for removing the detergent or phospholipid. Hence, DOC was present in the final product in amounts of 4 μg per dose. We find this acceptable to humans since the average serum concentration of DOC is 0.8 g/ml (43). Further, DOC (18) and phospholipid (44) in OMV seemed to contribute to the low pyrogenicity, despite the rather high LPS content of about 2 μg/dose. The side effects (45), immunogenicity (14,46) and protective effect (40) of this vaccine in humans have been presented.

Little variation between the sub-batches was found for the MenB-vaccine over a

two year period. Although minor quantitative differences were noted, these were reduced by pooling sub-batches before adsorption of the final vaccine onto $Al(OH)_3$. During the two years of production, no batch was excluded on the basis of quality control tests alone, but a few sub-batches were rejected mainly on the basis of environmental monitoring results.

At present multicomponent meningococcal vaccines like this OMV-vaccine are not fully characterized. Attention must therefore be paid to detailed characterization of the vaccine components and to assays designed to detect subtle batch-to-batch variations. These may or may not influence the efficacy or reactogenicity of the vaccine, but at this stage of vaccine development all parameters need to be carefully monitored.

The MenB-vaccine «Folkehelsa» has been shown to be efficacious, although not as protective as hoped for (14,40,46). Careful analysis of the data and biological material from the clinical trial may provide an important basis for better understanding of the nature of protective components. Such studies are in progress. A goal for future research is to obtain good surrogate tests for vaccine efficacy. Improved assays for interpretation of immunogenicity data should help strengthening the criteria by which such vaccines are judged in the future.

Aknowledgement

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DISCUSSION

Poolman:
Regarding the different lots that you have made, did you look for variation between the organism you put in and the organism that came out, with respect to quality and epitope change?

Fredriksen:
I cannot say very much about the topic at this stage. We have looked at the different batches with our present panel of monoclonal antibodies and polyclonal vaccince sera. If you have reagents that may characterize other vaccine components besides the major class proteins, we are very interested. For the class 5 protein, *e.g.*, we have only been checking the epitopes designated 5.5 and 5C.

Robbins:
Have you analysed the capsular polysaccharides in the final preparation?

Fredriksen:
We did some chemical B-PS measurements during the developmental process. B-PS was hardly detectable in the final product. We have not used this kind of analysis as a regular quality control. The production procedure we use does not selectively exclude B-PS. However, B-PS is soluble so it should be removed by the washings, but some may remain.
(Later an ELISA method with a monoclonal against B-PS was established. The level of B-PS in the vaccine was estimated by this method to be below 0.13 μg/ml; see Fredriksen *et al.*, the preceding report).

Frasch:
A couple of points: We have used a non-encapsulated variant, so therefore we did not have to worry about PS. Concerning the lipopolysaccharides; Dr. Tsai in our lab has shown that if you have free LPS versus membrane-bound LPS, membrane-bound LPS is about a hundred times less toxic than if the LPS is free in solution. So I think that is why the vaccine contains more LPS than you would normally think. I think it is critical that the vaccine is controlled by looking for intact vesicles. One last point; you said the vaccine was purified and maintained aseptically, but I did not see if a sterility test was among the QC-tests done?

Fredriksen:
The test for sterility is definitely one of our quality controls and it was included in the slide schematically presenting the QC-tests. To assure sterility, however, we use a predesigned environmental monitoring programme. The test for sterility is hardly of any value if done isolated from environmental control data.

Gotschlich:
Dr. Frasch and you have made a great point of the need for the vaccine to consist of vesicles. The question is, how many preparations failed to give you vesicles?

Fredriksen:
Every batch we looked at contained vesicles. But I am quite certain that for some OMP-vaccines, with less LPS and DOC, the vesicle formation can be a problem. I think it is an important point to consider when it comes to the formulation and antigen presentation. We do electron microscopy as a quality control. Upon storage the vesicle formation tends to differ and some aggregation can be observed.

Broome:
Have you ever identified any bad lots, either by mouse immunogenicity or by human immunogenicity, so that you can look back on their characteristics?

Fredriksen:
Some batches have been excluded, but we have not yet looked thoroughly into the data or analysed the biological material available.

Frasch:
One clarification: You said that the natural strain was positive for iron-regulated proteins. Was that in the strain or in the vaccine? Because you mentioned an 86k protein and sort of dropped it at that.

Fredriksen:
So far, some iron-regulated proteins have been identified in the strain. In the vaccine, minor bands with a molecular weigth of about 80kD can be seen in SDS-gels, but we do not know if these are the iron-regulated ones.